Heparin as an inhibitor of cancer progression

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Chapter outline:

1. Summary

Heparin is frequently used for treatment of cancer-associated thromboembolism. Accumulating clinical evidence indicates that cancer patients treated with unfractionated and low-molecular weight heparin survives longer that patients treated by other anticoagulants, especially patients in the early stage of a disease. Experimental analysis from a number of animal models constantly provides evidence about the ability of heparin to attenuate metastasis. The non-anticoagulant activity of heparin on metastasis includes the ability to inhibit cell-cell-interaction through blocking of P- and L-selectin, to inhibit extracellular matrix protease-heparanase, and to inhibit angiogenesis. This chapter summarizes the current experimental evidence on the biology of heparin during cancer progression with the focus on potential mechanism of heparin antimetastatic activity.

2. Heparin affects on cancer: clinical evidence

The close relationship between cancer and venous thromboembolism (VTE) has been recognized for a long time, and multiple risk factors during malignancy have been linked to hypercoagulability. Thromboprophylaxis with unfractionated heparin and low-molecular weight heparin (LMWH) has been used for management of hypercoagulable state in cancer patients, and LMWH is the recommended anticoagulant regimen by international guidelines [1, 2]. Many retrospective analyses of clinical data indicated that heparin treatment affects survival of cancer patients with various tumors, especially in patients with the early stage of a disease [3-5]. Based on these observations several prospective clinical trials has been performed to evaluate heparins for its anticancer potential [6-14]. The CLOT study was designed to study the effect of LMWH or an oral anticoagulant – Coumarin on prevention of venous thromboembolism in patients with solid tumors [6, 7]. While this study showed no overall effect on cancer progression, a significant increase in patient survival was detected in a subgroup of patients that were without metastasis at the beginning of the trial [6]. Furthermore the treatment with LMWH – Nadroparin was significantly more effective than the oral anticoagulant [7]. In a trial of patients with small cell lung cancer, the use of LMWH – Dalteparin in parallel to standard chemotherapy significantly prolonged median survival of patients when compared to patients with the chemotherapy only [9]. However, no significant difference was observed in a study of advanced cancer patients treated with Nadroparin for a prolonged time [12]. The Fragmin Advanced Malignancy Outcome Study (FAMOUS) trial evaluated the effect of Dalteparin in 374 patients with advanced malignancies treated for a year [8]. While in patients with originally poor prognosis no statistically significant increase in survival was detected, in patients with a better prognosis Dalteparin treatment resulted in a significant improvement of patient survival. The Malignancy and LMWH Therapy (MALT) trial of patients with advanced cancer of various origins demonstrated an improvement of survival when compared to a control group [11]. The subgroup analysis of patients with a

better prognosis in the MALT and FAMOUS studies further underlines the potential of heparins to affect earlier stages of cancer. There are several ongoing clinical trials with an aim to validate the effect of heparin on cancer progression in specific cancer types. Nevertheless, the available evidence about heparin activity in cancer patients obtained from several completed studies indicates that heparin seems to directly affect cancer progression.

3. Heparin attenuates metastasis in experimental models

The effect of heparin on cancer progression has been reported by a number of laboratories, most of which used a mouse model of experimental metastasis [reviewed in 15-17]. In the initial studies, application of unfractionated heparin attenuated metastasis of mammary carcinoma and melanoma cells [18, 19]. Despite many limitations of the experimental metastasis model, the direct application of tumor cells in the circulation allows a more detailed analysis of heparin inhibitory potential that is limited to a timely defined presence of heparin in the circulation. The application of heparin around the time of tumor cell injection resulted in attenuation of metastasis that was observed in the majority of studies using different tumor cell lines, a wide range of heparin doses and a variety of heparin preparations [see reviews 15-17]. A single dose of heparin effectively attenuated metastasis of human and mouse colon carcinoma and melanoma, mouse breast carcinoma and lung carcinoma. Application of heparin either 24 hours before, or after the tumor cell injection had no effect [19, 20]. Attenuation of metastasis was generally independent of the route of heparin application. While intravenous application of heparin around the time of tumor cell injection always led to a reduction of metastasis, subcutaneous or intraperitoneal application of heparin were mostly effective [16, 21-24]. The observed limited response to heparin has been likely due to variations in time and/or route of heparin application, amounts of applied heparins and differences in heparin preparations used [21-24]. In the majority of studies the amount of

heparin applied significantly exceeded the clinically used therapeutic dose. Nevertheless, recent studies provided evidence that heparin attenuated metastasis in two different mouse models also at clinically relevant concentrations [25, 26]. Taken together, a number of animal experiments convincingly support the ability of heparins to attenuate metastasis. Mouse model of experimental metastasis proved to be instrumental for narrowing down the potential mechanism underlying the heparin activity during cancer progression (see below). However, this model had several limitations which do not allow to analyze the various stages of metastasis or the root of metastasis (lymphatic versus hematogenous). Heparin has been shown to attenuate metastasis only in one study with a spontaneously metastatic mouse model, while tumor growth remained largely unaffected [27]. Further studies in spontaneously metastatic mouse models are required to confirm the findings from experimental models with the primary focus on an identification of the potential mechanism of heparin activity in this process.

4. Diverse biological activities of heparin

Heparin is a complex mixture of natural glycosaminoglycans that contains a variety of biological activities. Heparin molecules are long unbranched polymers built of disaccharide repeats consisting of glucosamine and iduronic/galacturonic acid with a high degree of sulfation. Heparin is found in granules of mast cells that line blood vessels and are also present in mucosal tissues. Clinical preparations of heparin are of porcine and bovine origins that are adjusted according to their anticoagulant activity. The molecular weight of an unfractionated heparin ranges between 12,000 – 14,000 Da. The partially depolymerized and fractionated LMWH consist of fragments with an average size of 5,000 Da. Although LMWH preparations differ from each other depending on the preparation method, chemical or enzymatical depolymerization, their pharmacokinetical parameters are comparable [28]. The

anticoagulant activity of heparin is defined by its ability to bind antithrombin III. A distinct pentasaccharide structure has been identified as the active site of heparin [29]. But this pentasaccharide represent only a small fraction of heparin preparations. Heparin binding to antithrombin alters its conformation and thereby accelerates its antithrombotic activity. The heparin –antithrombin complex binds to an active factor X with a high affinity thereby inactivating the factor Xa. While unfractionated heparin and LMWH have comparable anti Xa activities, they differ in their ability to inhibit factor IIa [30]. Binding of factor IIa requires a formation of a complex around a longer chain of the heparin fragment than is usually not present in LMWH preparations.

Heparin preparations are mixtures of heterogenous polysaccharides containing a large variety of biological activities, which makes the identification of the heparin role during cancer progression rather complicated. There is accumulating evidence that heparin, apart from its anticoagulant activity, can block P- and L-selectin, affect activity of growth factors and cytokines, release tissue factor pathway inhibitor (TFPI), inhibit heparanase and angiogenesis, alter interactions with integrins and modulate protease activity and thereby the composition of extracellular matrix [15, 29, 31-35]. While any of these activities may affect cancer progression (Figure 1), the experimental evidence from a number of studies strongly indicates that heparin affects early events in the metastatic cascade.

5. Potential mechanisms of heparin affecting cancer progression

Metastasis is a process during which invasive tumor cells enter the circulation, avoid immune responses, attach to the endothelium of distant organs, extravasate from the vasculature and establish metastatic foci. The common observation in most animal studies that a single dose of heparin before tumor cell injections attenuates metastasis strongly indicates that heparin affects the initial phase of hematogenous metastasis. This observation is further supported by

the fact that the half-life of heparin in circulation ranges between four to six hours [36, 37]. Despite the relatively short time of heparin availability in the circulation, heparin significantly modulates the metastatic capacity of tumor cells while they are still in the circulation. Thus, heparin is affecting immediate processes upon tumor cell injection into the circulation like inhibition of P-and L-selectin-mediated interactions and affecting coagulation. Consequently, the inhibitory affect of heparin on angiogenesis or extracellular matrix remodeling are unlikely to be affected during this period.

6. Anticoagulant activity of heparin

Cancer patients are prone to thrombotic complications and LMWH and UFH are used as an effective therapeutic anticoagulant treatment or as a prophylaxis [38]. A retrospective analysis of a number of clinical trials, where heparin treatment was compared with other anticoagulant regimen (e.g. Coumarin), indicated that heparin exerts activity beyond its anticoagulation [29, 39, 40]. Heparin effect on cancer progression has been confirmed in animal models and has been found to be additional to its anticoagulant activity [15, 17, 41]. The use of modified heparins with minimal or no anticoagulant activity led to attenuation of metastasis in a number of various mouse models using different cancer cell lines, indicating that heparin affects metastasis largely in an anticoagulant independent manner [19, 21, 22, 27, 37, 42-44]. The use of an anti-thrombin inhibitor, hirudin, have been shown to reduce metastasis but the used amount of hirudin was strongly exceeding clinically relevant concentrations [45, 46]. The identification of a pentasaccharide reponsible for the antithrombin inhibitory activity in the heparin sequence enabled to evaluate the anti-cancer activity of heparin [47]. While the synthetic pentasaccharide – fondaparinux did not affect cancer progression, LMWH effectively attenuated metastasis at clinically tolerable levels [25, 26] However, the antimetastatic activity of LMWH has been found to vary among different preparations.

Heparins have also the ability to release tissue factor pathway inhibitor (TFPI) from the blood vessel walls and thereby affect metastasis [35]. Application of LMWH – Tinzaparin led to a reduction of metastasis. Intravenous injection of recombinant TFPI at the time of tumor cell injection significantly attenuated metastasis of melanoma [35, 48]. The extent of heparin mediated TFPI release and its contribution to inhibition of cancer progression requires further in vivo investigation in the context of other non-anticoagulant activities of heparin.

7. Inhibition of heparanase

Tumor cells invasion is an essential step of cancer progression that is associated with an ehnaced capability of tumor cells to degrade extracellular matrix components, including heparin sulfate proteoglycans, collagens and fibronectin [49]. Tumor cells produce several hydrolytic enzymes including matrix metalloproteinases and heparanase, and their expression correlates with their metastatic potential. Heparanase is an endoglycosidase that cleaves heparin sulfate. Elevated heparanase expression has been detected in association with cancer progression of several carcinomas including colon, liver, pancreas, bladder, breast and prostate carcinoma as well as leukemia and multiple myeloma [50-52]. Heparanase overexpression in human tumors confers accelerated tumor growth and invasive phenotype in experimental animals [52]. Silencing of the heparanase gene in cancer cells resulted in a reduced angiogenesis, tumor growth and metastasis, thereby directly linking heparanase activity to cancer progression [53]. Several studies provided evidence that heparin inhibits heparanase activity *in vitro* and that modified heparins without anticoagulant activity but heparanase inhibitory activity attenuated metastasis of melanomas and multiple myelomas [19, 54, 55]. Furthermore, sulfated oligosaccharides and laminarin sulfate with heparanase inhibitory activity attenuated experimental metastasis of melanomas and breast carcinomas [56, 57]. Heparanase has been shown to regulate the function of heparan sulfate and thereby

affect tumor growth and metastasis in multiple myeloma [54, 58]. Shedding of heparan sulfate proteoglycan syndecan-1 from myeloma cells into their microenvironment is mediated by heparanase and is critical for the cancer progression [54]. The use of a heparanase-specific modified heparin NA-ROH (100%-N-acetylated, 25% glycol-split) attenuated primary tumor growth and metastasis [54, 55]. Recently, the effect of NA-ROH heparin has been tested also in colon carcinomas, where no attenuation of metastasis was observed [37]. Therefore, inhibition of heparanase leads to attenuation of metastasis only in tumor cells expressing this enzyme.

8. Selectins as potential targets of heparin

Unfractionated heparin has been found to be an efficient ligand for P- and L-selectin [59, 60]. Selectins are vascular cell adhesion molecules involved in adhesive interactions of platelets, leukocytes and endothelial cells within the blood circulation. The physiological role of selectins has been described in hemostasis, inflammation and immune response [61, 62]. Selectins mediate the initial interactions of leukocytes with the vascular endothelium. The rapid and reversible interactions between selectins and their carbohydrate ligands enable leukocyte rolling that may result in firm adhesion. There are three members of the selectin family: L-, P- and E-selectin. While L-selectin is constitutively expressed on the almost all leukocyte populations, P-selectin is constitutively expressed in the secretory granules of platelets and endothelial cells. Upon activation, P-selectin is rapidly expressed on the cell surface of both endothelial cells and platelets, thereby initiating cell adhesion. E-selectin expression on endothelial cell surfaces requires de novo transcription, thus occurs several hours after activation [63]. Selectin ligands usually carry sialylated, fucosylated lactosamine oligosaccharide structures containing the terminal tetrasaccharide sialyl Lewis^{x} or its isomer sialyl Lewis^{a} (sLe$^{x/a}$) [63, 64]. Additional sulfation of the glycan itself or of the protein

backbone in close proximity to the oligosaccharide further enhances the specific recognition by P-and L-selectin [65]. In addition to this common selectin ligand, P- and L-selectin can efficiently bind to heparin and sulfated galactosylceramide – sulfatides [59, 60, 66]. The ability of selectins to recognize a variety of different carbohydrate structures indicates that the lectin domain of selectins binds to a certain carbohydrate "patch" [67].

9. Carcinomas, heparin and hematogenous metastasis

Hematogenous metastasis is the common route of cancer spread for epithelial cancer carcinoma. Epithelial cells are lining up the lumen of hollow organs that are covered by mucins. Cell-surface-mucins as well as soluble mucins secreted on apical sites of the epithelium contribute to protection of the organs from pathogens. Mucins are high molecular weight molecules that contain a large portion of O-linked glycan structures [68, 69]. Carcinoma cells show altered cell surface glycosylation with an enhanced presence of sLe^x, sLe^a, Tn and sialyl-Tn oligosaccharide structures on mucins [68, 69]. The positive correlation between $sLe^{x/a}$ expression and poor prognosis, due to metastasis, has been demonstrated in colon, gastric, lung, prostate, renal and breast cancers [70-76]. The correlation between selectin ligand expression and poor prognosis for carcinoma patients indicates the potential involvement of selectins in cancer progression. Moreover, the presence of carcinomas carrying selectin ligands in blood circulation makes it possible to mediate interactions with selectins on platelet, leukocytes, and endothelium thereby leading to metastasis [16].

10. P- and L-selectin facilitate metastasis

Hematogenous metastasis is a process consisting of several events enabling the tumor cell to leave the primary tumor, enter the blood circulation, evade immune responses, adhere to the vascular cells of distant organs and exit from circulation and form the new metastatic lesion.

Of these events, cell-cell interactions leading to tumor cells adhesion to the endothelium of distant organs appear to be critical for this process [77, 78]. The rapid expression of P-selectin on platelets and endothelium upon activation and the constant presence of L-selectin on leukocytes further supported the hypothesis that selectins may facilitate metastatic initiation. The recent evidence obtained in P- and/or L-selectin deficient mice confirms the contribution of these early response receptors to metastasis [20, 36, 79-82]. Attenuation of metastasis was observed in the absence of P-selectin both with carcinomas and melanoma cells expressing selectin ligands [20, 36, 37, 80]. The absence of P-selectin led to a reduction of tumor seeding to the lung vasculature that was associated with a reduced platelet-tumor cell emboli formation [36]. Similarly, the reduction of selectin ligands on tumor cells caused a decrease in platelet-tumor cell emboli formation and attenuation of metastasis [36, 80, 83]. The platelet-tumor cell emboli formation seems to protect tumor cells from elimination by NK cells and this process is largely P-selectin dependent [84]. Bone marrow reconstitution of lethally irradiated P-selectin deficient mice has shown that also the endothelial P-selectin expression contributes to metastasis [20].

Attenuation of metastasis was observed also in L-selectin deficient mice, strongly implicating leukocytes to be active facilitators of this process [80, 81]. L-selectin-mediated recruitment of leukocytes to vascular tumor cells was associated with an enhanced expression of L-selectin ligands surrounding tumor emboli [81]. Intravenous injection of a function blocking L-selectin antibody resulted in attenuation of metastasis [81].Although the mechanism of leukocyte contribution to metastasis requires further investigations, the current evidence indicates that leukocytes may potentiate tumor cell extravasation [85-88]. The individual absence of P- or L-selectin significantly attenuated metastasis of carcinoma cells, but in the P- and L-selectin double deficient mice virtually no metastasis was observed [80]. These

observations strongly indicate a synergistic effect of both selectins during initiating steps of metastasis.

The over-expression of E-selectin in the liver was shown to divert metastasis to this organ [89]. In another study, experimental liver metastasis has been shown to be blocked by E-selectin blocking antibody [90, 91]. In an experimental metastasis model where mice injected with IL-1 where co-injected with tumor cells together with soluble E-selectin reduction of metastasis to the lungs has been observed [92]. However, the cytokine-induced E-selectin expression may not reflect the natural process of metastasis. Interestingly, in the absence of E-selectin no attenuation of experimental metastasis to the lungs was observed [82]. Due to the delayed cell-surface expression, E-selectin may not be affecting the very early steps of naturally occurring metastatic processes. Therefore, the presence of early response receptors P- and L-selectin in the blood circulation suggests the implication of endothelium, platelets and leukocytes in metastasis that can be inhibited by heparins.

11. Heparin inhibits P and L-selectin-mediated interactions

Heparin was shown to attenuate metastasis in a number of different animal models when applied at the time of tumor cells injection [reviewed in 16, 17]. To address the potential of heparin in the process to affect tumor cell invasion and migration into the blood vessels, spontaneous metastatic models are necessary. Nevertheless, the widely used experimental metastasis model enables to characterize the molecular mechanisms underlying the effect of heparin on the initiation phase of metastasis. The antimetastatic activity of heparin was analyzed in P- and/or L-selectin deficient mice [20, 36, 37, 80, 81, 93]. Injection of heparin shortly before tumor cell injection attenuated metastasis in wild type (wt) mice to similar levels as observed in P-selectin deficient mice (Psel-/-) [20, 36, 80]. Heparin injection shortly

before tumor cell injection further attenuated metastasis in L-selectin deficient mice (Lsel-/-) [80, 81]. This heparin administration efficiently inhibited platelet-tumor cell emboli formation that was shown to be largely P-selectin dependent [36]. No additional reduction of metastasis was observed by heparin in Psel-/- mice [20, 36, 80]. Attenuation of metastasis was also achieved with heparin derivatives without anti-coagulant activity together with the finding that heparin showed no effect in Psel-/- mice, heparin treatment at time when tumor cell are in circulation likely influence P-selectin mediated interactions during early steps of metastasis [37].

The additional attenuation of metastasis achieved by heparin treatment at time of tumor cell injection in Lsel-/- mice indicates that P-selectin-mediated platelet aggregation precedes the involvement of L-selectin, thereby leukocytes in this process [81]. Accordingly, heparin treatment several hours post-tumor cell injection – late heparin (6-12 h) further attenuated metastasis in Psel-/- mice, while had no further effect in Lsel-/- mice [81]. The late heparin treatment in wt mice resulted in attenuation of metastasis to a similar extent as observed in Lsel-/- mice. The final evidence that heparin affects primarily P- and L-selectin mediated interactions came from the P- and L-selectin double deficient mice [80]. Heparin treatment, either shortly before or later, after tumor cell injection had no further effect on metastasis. Interestingly, prolonged treatment of P- and L-selectin double deficient mice with high doses of UFH further reduced metastasis, indicating heparin affect on other mechanisms of metastatic progression [93]. Recently, different clinically used LMWH preparations have been shown to attenuate metastasis by inhibition of selectin [25, 26].

12. Mechanism of heparin action during metastasis

Hematogenous metastasis is directly or indirectly responsible for most cancer-related deaths. Accumulating evidence from a number of laboratories indicates that heparin attenuates

experimental metastasis of various cancer cells, as long as dose of heparin or its derivative reaches the clinically used concentration and is applied around the time when tumor cells are still in the circulation [reviewed in 16, 17]. There have been only two exceptions, where heparin did not affect metastasis applied around the time of tumor cell injection [22, 23].Heparin treatment of mice either 1 day before or 1 day after tumor cell injection did not affect metastasis [19, 20]. Despite the large variation of heparin doses applied, attenuation of metastasis has been observed. Human P-selectin sensitivity is about ten fold higher when compared to mouse P-selectin [36]. The affectivity of non-anticoagulant heparins as metastatic inhibitors further excludes the possibility that the inhibition of prothrombotic activities is the main mechanism of heparin anti-cancer activity [19, 27, 37]. Basically, all published studies using heparin or its derivatives in experimental metastasis models are in agreement with the potential inhibition of P- and/or L-selectin function. While other biological activities can clearly contribute to anti-metastatic activity of heparin, the relatively short time of heparin presence in the circulation makes them less likely to be affective in this context. Further analyses of heparin as an inhibitor of cancer progression are required and should validate heparin as an inhibitor of cancer progression in spontaneously metastatic mouse models. Analysis of P-and/or L-selectin inhibitors during metastasis might complement the knowledge about this process. Yet, the very nature of heparin, carrying not only selectin-inhibitory activity but also the activity towards heparanase, binding of cytokines, TFPI release or modulatory activities on extracellular matrix, might prove beneficial over a single targeting of selectins. Together with the currently available clinical evidence from already performed clinical trials as well as ongoing trials supports the future evaluation of heparin as a potentially first treatment specifically for cancer metastasis.

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Figure Legend

Figure 1 Diverse biological activities of heparin potentially affecting cancer progression. Heparin may affect any of these activities and thereby inhibit cancer progression. Many of these activities have been shown in vitro and the individual contribution to attenuation of cancer progression remains to be determined in vivo.

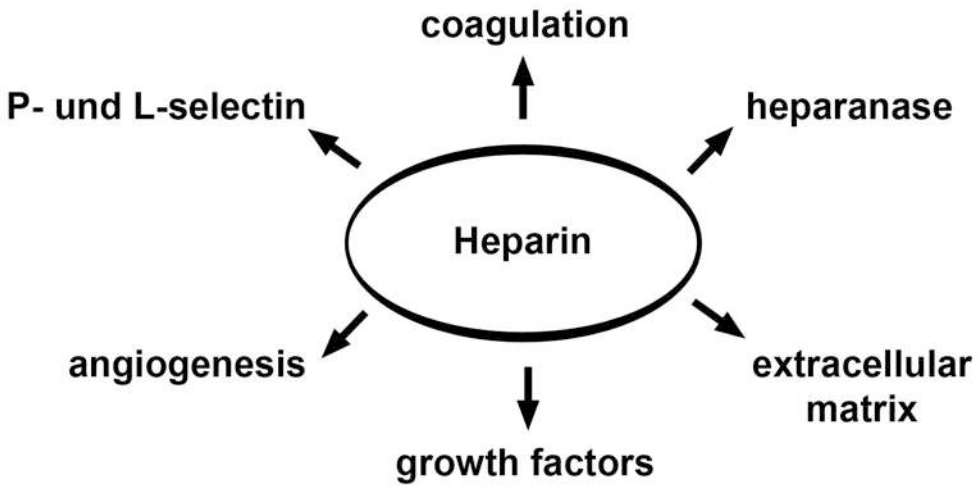
coagulation
P- und L-selectin
heparanase
Heparin
angiogenesis
extracellular matrix
growth factors

Figure 1